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PRINCIPAL INVESTIGATOR:
Ignacio I. Wistuba, M.D.

CONTRACTING ORGANIZATION:
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Houston, TX 77030

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6. AUTHOR(S)

Humam Kadara, Ph.D.
Ignacio Wistuba, MD

E-Mail: iiwistuba@mdanderson.org

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Houston, TX 77030

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14. ABSTRACT
During our third year, we found that the transcriptomic architecture of the adjacent airway field cancerization in NSCLC is comprised of markers that identify lung cancer among smokers as well as gradient expression patterns that recapitulate tumor profiles, and thus, provides additional insights into NSCLC biology and the development of molecular tools for the detection of the malignancy. Furthermore, we studied in detail the functional roles of *LAPTM4B*, a putative oncogene that we identified as a field cancerization marker, in lung cancer pathogenesis. We found that *LAPTM4B* promotes growth of lung cancer cells in soft agar, is associated with smoking and worse overall survival, protects cells from serum deprivation-induced growth inhibition and positively controls the autophagic response and the NRF2 transcription factor during cellular stress. Furthermore, using a novel molecular spatial mapping approach, we identified gene sets, in patients with lung cancer, which decrease in enrichment with larger distance from the tumor as well as those that persist up to the nasal epithelium. These data point to specific tumor-associated profiles that are enriched in the nasal epithelium and, thus, comprise readily accessible markers for lung cancer detection that will be further refined and validated in aim 3 of the study.

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INTRODUCTION

Lung cancer continues to be the leading cause of cancer-related death in both men and women in the United States (1). The majority of lung cancers are non-small cell lung cancers (NSCLCs) that include squamous cell carcinomas (SCCs) and adenocarcinomas (2) Lung cancer mortality is high in part because most cancers are diagnosed after regional or distant spread of the disease had already occurred and due to the lack of reliable biomarkers for early detection and risk assessment (2). The identification of new effective early biomarkers will improve clinical management of lung cancer and is linked to better understanding of the molecular events associated with the development and progression of the disease.

It has been suggested that histologically normal-appearing tissue adjacent to neoplastic lesions display molecular abnormalities some of which are in common with those in the tumors (3). This phenomenon, termed field of cancerization, was later shown to be evident in various epithelial cell malignancies, including lung cancer (4, 5). Loss of heterozygosity (LOH) events are frequent in cells obtained from bronchial brushings of normal and abnormal lungs from patients undergoing diagnostic bronchoscopy and were detected in cells from the ipsilateral and contralateral lungs (6). More recently, global mRNA expression profiles have been described in the normal-appearing bronchial epithelium of healthy smokers (7) including those that were diagnostic of lung cancer (8). In addition, modulation of global gene expression in the normal epithelium in health smokers is similar in the large and small airways and the smoking-induced alterations are mirrored in the epithelia of the mainstem bronchus, buccal and nasal cavities (9).

In this program, in Specific Aim 1, high-throughput microarray mRNA expression analyses were performed on cytologically controlled lung tumors, airways with varying distances from corresponding tumors and normal lung tissues obtained at lobectomy procedures. Towards this aim, we are comparing and contrasting global gene expression patterns across all the specimens from the entire field and corresponding NSCLC tumors as well as understanding the transcriptomic architecture of the adjacent-to-tumor field of cancerization with respect to spatial proximity from tumors. Moreover, and in collaboration with the Initiating PI (Dr. Steve Dubinett) and other Partnering PIs (Dr. Avrum Spira and Dr. Pierre Massion) we are currently performing RNA-sequencing and microarray profiling of nasal epithelia, airway epithelial cells collected from both bronchoscopy and lobectomy specimens as well as of corresponding tumors (NSCLC patients) or benign lesions (cancer-free individuals). In this analysis, we are mapping field effects that transverse the normal-appearing bronchus adjacent to lesions up to the nasal epithelium and pointing to tumor-associated field cancerization profiles that remain enriched up to the nasal epithelium. In Specific Aim 3, we will use expression signatures and biomarkers derived from this aim of the study to develop and test airway-based biomarkers capable of diagnosing lung cancer in current or former smokers using minimally invasive sites.

This report details the progress made during the third year of research. This report will also be included in the comprehensive annual report to be prepared and submitted by Dr. Steven Dubinett (UCLA) as the Initiating PI.

MD Anderson Cancer Center (Dr. I Wistuba) PROGRESS REPORT

Molecular Profiles for Lung Cancer Pathogenesis and Detection in U.S. Veterans

Specific Aim 1: To increase our understanding of the molecular basis of the pathogenesis of lung cancer in the "field cancerization" that develops in current and former smokers.

Summary of Research Findings

A. Gene expression analysis of bronchial epithelial samples obtained from lobectomy specimens from NSCLC patients (*Field Cancerization Study*)

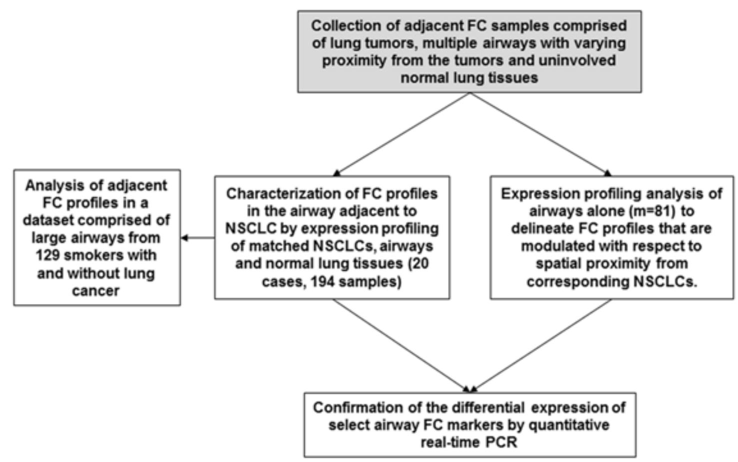

Figure 1. Schematic of the transcriptomic analysis of the airway Field cancerization in NSCLC. Field cancerization samples comprised of matched lung tumors, multiple cytologically controlled normal airways with varying proximity from tumors and uninvolved normal lung tissues were obtained from 20 resected early-stage (I-IIIA) NSCLC specimens. Gene expression profiling of all the samples (n=194) was performed to characterize global cancerization profiles (differentially expressed in the same direction between both tumors and airways compared to normal lung tissues) in the normal-appearing airway adjacent to NSCLCs (site-independent analysis). Adjacent field profiles were then analyzed by GSEA in a set of large airways of smokers (n=129) with and without lung cancer from BU (PI: Avrum Spira) to identify profiles that can distinguish lung cancer among smokers. Global expression profiling was also employed to delineate FC profiles that are modulated in the airway with respect to proximity from corresponding NSCLCs (site-dependent analysis). QRTPCR was used to confirm the differential expression of select airway markers.

We sought to characterize the yet unknown global molecular and adjacent airway field cancerization in early-stage NSCLC. We performed whole-transcriptome expression profiling of resected early-stage (I-IIIA) NSCLC specimens (n=20) with matched tumors, multiple cytologically controlled normal airways with varying distances from tumors and uninvolved normal lung tissues (n=194 samples) using the Affymetrix Human Gene 1.0 ST platform. Samples were obtained from patients who did not receive neoadjuvant therapy undergoing lobectomy or pneumonectomy procedures under an MD Anderson institutional review board (IRB)-approved protocol. Mixed-effects models were used to identify differentially expressed genes among groups. Ordinal regression analysis was performed to characterize site-dependent airway expression profiles. A schematic of the study's design is represented in **Figure 1**. **Data from the gene expression analysis have been described and detailed in the previous annual report (Year 2).** We identified differentially expressed gene features (n=1661) between NSCLCs and airways compared to normal lung tissues. We then examined the expression of the adjacent airway FC profile in a cohort comprised of 129 large airway samples from smokers with and without lung cancer. This analysis identified a subset (n=299), following gene set enrichment analysis, that significantly (*P* <0.001) and concordantly clustered large airways of healthy smokers from airways of lung cancer patients.

We also identified a cassette of gene features (n=422) that were significantly and progressively differentially expressed in airways by distance from tumors (**Figures 2A and 2B**). Notably, when we examined the 422 gene features in NSCLCs and paired normal lung tissues, we found that

291 of the 335 genes that were increased and 53 of the 87 that were decreased in airways with shorter distance from tumors were also up-regulated and down-regulated, respectively, in NSCLCs compared to normal lung tissues and that a field cancerization score quantifying the site-dependent effect in the airway was congruently modulated between NSCLCs and normal lung (**Figures 2C and 2D**). Our findings suggest that the adjacent airway field of cancerization harbors profiles and pathways that are both site-independent as well as gradient and localized with respect to nearby tumors and that may point to new molecular tools for detection of NSCLC and further inform of the molecular pathology of the malignancy. These data have been submitted as an abstract in the past AACR and have been submitted for publication and currently under revision (**see Reportable Outcomes**).

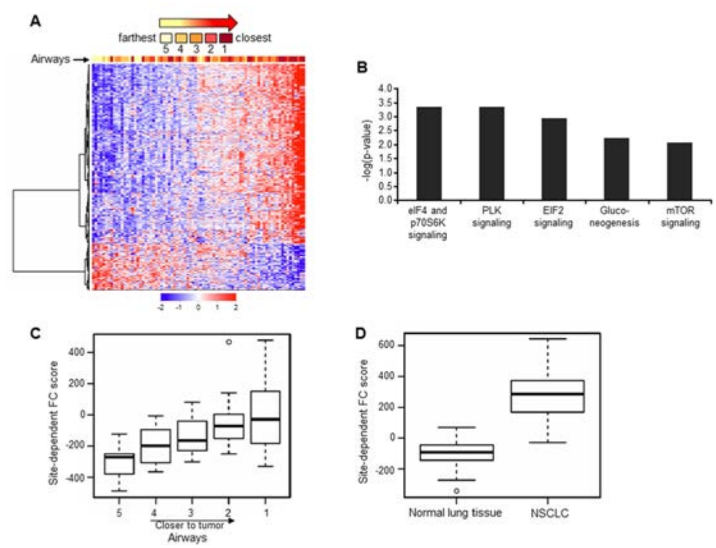

Figure 2. Analysis of airway expression profiles by distance form corresponding NSCLCs. A. Ordinal logistic regression analysis of airways was performed and identified 422 gene features with significant differential expression in the airway with respect to tumor proximity (FDR <5%). **B**. Functional pathways analysis by IPA of the site-dependent differentially expressed genes. Box plots depicting site-dependent FC score in airways (**C**) and between corresponding paired NSCLCs and normal lung tissue (**D**).

B. Expression validation and functional studies

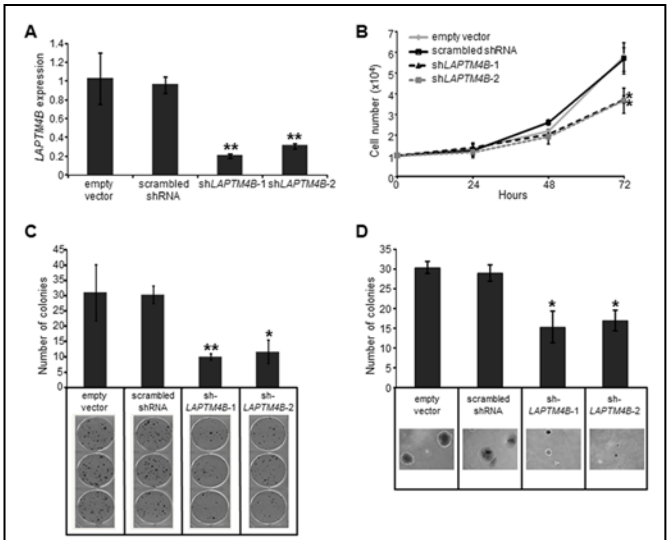

Figure 3. Stable knockdown of *LAPTM4B* reduces lung cancer cell growth and colony formation. A. QRTPCR analysis of Calu-6 cells stably transfected with empty vectors or vectors containing scrambled shRNA or *LAPTM4B*-specific shRNA. **B**. Trypan blue exclusion method count of the stably transfected cells. Anchorage-dependent (**C**) and –independent (**D**) colony formation assay of the stably transfected Calu-6 cells was performed. Representative images of cell colonies are depicted in the lower panels. Error bars indicate standard deviation. *, P <0.05; **, P <0.001 by the Student's *t* test.

We performed QRTPCR analysis of select field cancerization markers including lysosomal protein transmembrane 4 beta (*LAPTM4B*), which was among the top 5 FC markers with increased expression in airways with respect to tumor proximity. **As detailed in our previous annual report (Year 2)**, QRTPCR confirmed microarray data and demonstrated that 1) *LAPTM4B* was significantly increased in NSCLCs and in airways with shorter distance from tumors; 2) *LAPTM4B* was significantly increased in immortalized lung epithelial cells compared to normal bronchial cells and 3) transient knockdown of *LAPTM4B* expression in immortalized and malignant lung epithelial cell lines significantly reduced cell growth. We sought to further confirm the role of *LAPTM4B* in lung cancer cell growth by generating sub-lines with stable knockdown of the gene in the Calu-6 cell line with high basal level of *LAPTM4B*. Stable knockdown of *LAPTM4B* significantly suppressed *LAPTM4B* (P <0.001) expression (**Figure 3A**) concomitant with significantly reduced cell growth (*P* <0.05) (**Figure 3B**) and anchorage-dependent (**Figure 3C**) and -independent colony number (all *P* <0.05) (**Figure 3D**). These data demonstrate that *LAPTM4B* is a positive mediator of malignant lung epithelial cell growth and phenotype. Moreover, these findings are proof of principle that characterization of field cancerization markers can lead to the identification of otherwise unknown mediators of lung cancer molecular pathogenesis. These data have also been submitted as an abstract in the past AACR and have been submitted for publication and currently under revision (**see Reportable Outcomes**).

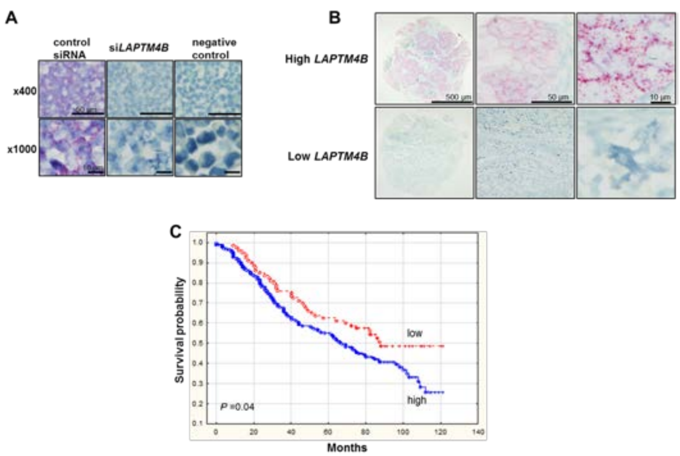

Figure 4. Analysis of *LAPTM4B* mRNA expression in lung cancer histological tissue specimens. A. Assessment of *LAPTM4B* mRNA by ISH was performed in blocks ofCalu-6 cell pellets transfected with control or *LAPTM4B*-specific siRNA. **B.** ISH was performed on a tissue microarray of 459 NSCLC FFPE specimens. C. Survival analysis based on *LAPTM4B* expression was performed using the Kaplan-Meier method for survival probability and the log-rank test.

We then sought to examine the expression of *LAPTM4B* in NSCLC formalin-fixed paraffin embedded (FFPE) histological tissue specimens. We opted to assess *LAPTM4B* transcript expression by *in situ* hybridization (ISH) due to limited antibodies available for immunohistochemical analysis of the protein product coding for this relatively understudied gene. We employed the QuantiGene 2.0 kit and QuantiGene View (QGV) RNA ISH tissue assay from Affymetrix (Santa Clara, CA) according to the manufacturer's instructions. The assay comprised singleplex probe for *LAPTM4B* based on the oncogene's transcript reference sequence from the NCBI (NM_018407). We first tested the hybridization assay in blocks of Calu-6 cell pellets (**Figure 4A**). The ISH assay showed abundant *LAPTM4B* mRNA expression in Calu-6 cells transfected with control siRNA (**Figure 4A** , left) compared to cells transfected with *LAPTM4B*-specific siRNA (**Figure 4A**, middle) and no reactivity in cells after omitting the probe (**Figure 4A**, right). These data demonstrate reliability of this assay to detect specific *LAPTM4B* expression and at variable levels. We then applied the ISH assay to study *LAPTM4B* expression in NSCLC FFPE histological tissue specimens (303 adenocarcinomas and 156 SCCs) and found that *LAPTM4B* expression was confined to epithelial tumor cells and absent in the stroma (**Figure 4B**). *LAPTM4B* expression was then quantified and statistically analyzed in association with various clinicopathological information including clinical outcome. We found that LAPTM4B mRNA by ISH was significantly higher in males, older patients and notable in smokers (all *P* <0.05). Additionally, higher (greater than the median) *LAPTM4B* mRNA expression was significantly associated with worse overall survival (*P* <0.05 of the log-rank test) in comparison to lower *LAPTM4B* mRNA in lung adenocarcinoma patients (n=303) (**Figure 4C**). These findings suggest that *LAPTM4B* field cancerization marker is associated with smoking and poor clinical outcome in the pathogenesis of human lung cancer.

We then determined to further examine the role of *LAPTM4B* field cancerization marker and putative oncogene in lung cancer pathogenesis. Earlier reports by others have demonstrated that *LAPTM4B* mediates breast cancer cell survival following metabolic and genotoxic stress (10-12). We were prompted to examine the relevance of *LAPTM4B* expression to effects of nutrient deprivation in lung cancer cells. We compared and contrasted the effects of serum

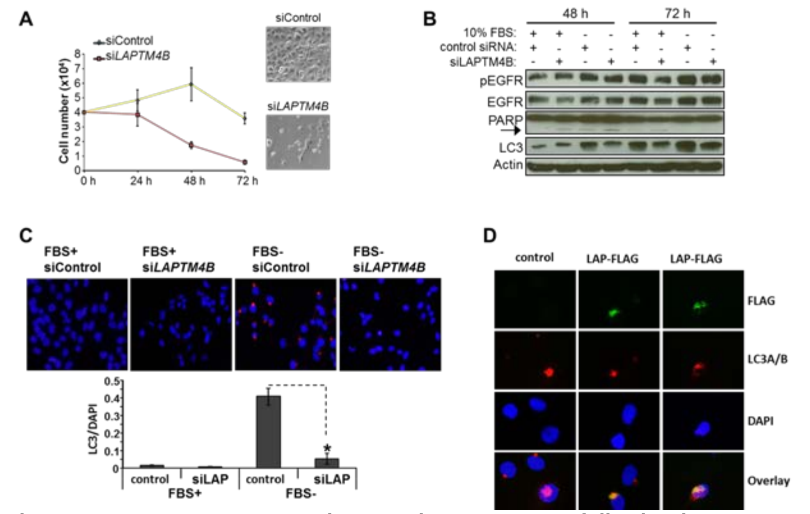

Figure 5. LAPTM4B promotes the autophagy response following lung cancer cell nutrient deprivation. A. Trypan blue exclusion-based cell count of cells transfected with control or *LAPTM4B*-specific siRNA. Cells were incubated in medium without FBS 24 h following transfection with siRNA. **B**. Western blotting analysis of cells similarly treated and transfected as in **A**. **C**. ICC analysis using a polyclonal antibody against LC3. **D**. ICC analysis of serum-starved cells transfected with control and FLAG-tagged *LAPTM4B* over-expression vectors.

starvation on cells transfected with control and *LAPTM4B*-specific siRNA. RNA interference-mediated knockdown of *LAPTM4B* significantly and largely augmented cell growth inhibition induced by serum starvation (**Figure 5A**). In addition, western blotting analysis demonstrated that knockdown of *LAPTM4B* alone in basal conditions decreased phosphorylation of the epidermal growth factor receptor (EGFR) proto-oncogene (**Figure 5B**). Additionally, knockdown of *LAPTM4B* increased serum starvation-induced cleavage of poly (ADP) ribose polymerase (PARP), indicative of augmented apoptosis induction. Notably, knockdown of *LAPTM4B* expression nearly abrogated the induction of the autophagy marker, LC3 (10), by serum starvation (**Figure 5B**). We then determined to quantify the levels of LC3 protein by immunocytochemical (ICC) analysis. Knockdown of *LAPTM4B* significantly attenuated (8-fold) LC3 induction by 48 h of serum starvation (*P* <0.001) (**Figure 5C**). We then performed ICC in serum-starved cells transfected with control vectors as well as a vector over-expressing a FLAG-tagged *LAPTM4B* construct. This analysis demonstrated that following serum starvation, LAPTM4B protein and LC3 co-localize intracellularly (**Figure 5D**). It is worthwhile to note that these findings are in accordance with earlier reports by others that pointed to the crucial role of *LAPTM4B* in the stability of the autophagosome in breast cancer cells (10, 11).

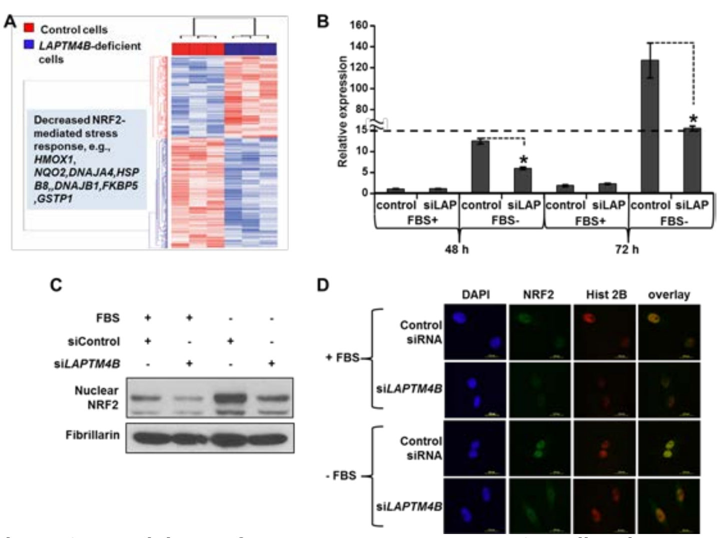

Figure 6. Knockdown of *LAPTM4B* attenuates NRF2-mediated stress response following serum deprivation. Cells transfected with control and *LAPTM4B*-specific siRNA and in the absence and presence of FBS were analyzed by global expression profiling (**A**), QRTPCR for *HMOX1* expression (**B**), western blotting for assessment of NRF2 levels in nuclear fractions (**C**) and by ICC to determine co-localization of NRF2 with the nuclear marker histone 2B. Error bars represent standard deviation. *, *P* <0.05.

We then determined to further understand the mechanisms that underlie augmented growth inhibition of lung cancer cells by decreased or absent *LAPTM4B* expression following serum deprivation. We performed global expression profiling, using the Human Gene 1.0 ST platform (Affymetrix), to compare and contrast the effect of serum starvation on the transcriptome of cells transfected with control and *LAPTM4B*-specific siRNA (**Figure 6A**). Expression data were normalized by RMA (13) and log (base 2) transformed. Gene features differentially modulated in the presence and absence of FBS between cells transfected with control and *LAPTM4B*-specific siRNA were determined by a mixed-effects model and a false discovery rate (FDR) cut-off of 5%. Ratios for the levels of each differentially expressed gene in the absence of serum over those in the presence of serum were determined. The ratios were then analyzed by clustering among and control and *LAPTM4B* deficient cells (**Figure 6A)**. Functional pathways analysis predicted significantly decreased activation of the nuclear factor erythroid 2-like 2 (NFE2L2 also known as NRF2)-mediated response (14) following serum withdrawal in *LAPTM4B* deficient cells (**Figure 6A**). We then performed QRTPCR analysis of heme oxygenase 1 (*HMOX1*) which is known to be transcriptionally modulated by *NRF2* (15) and was found by our array analysis to be decreased following knockdown of *LAPTM4B* (**Figure 6A**). QRTPCR analysis demonstrated that *HMOX1* mRNA levels were significantly increased after 48 h and 72 h of serum withdrawal (**Figure 6B**). Knockdown of *LAPTM4B* significantly attenuated *HMOX1* induction by serum withdrawal (**Figure 6B**). We then sought to assess the effect of *LAPTM4B* expression on the NRF2 transcription factor itself. In accordance with QRTPCR analysis of its downstream target *HMOX1*, knockdown of *LAPTM4B* attenuated nuclear accumulation of NRF2 protein by serum starvation as evident by western blotting of nuclear cell fractions (**Figure 6C**) and by ICC analysis of the co-localization of NRF2 with the nuclear marker histone 2B (**Figure 6D**). Furthermore, reciprocal effects were observed in cells transfected with *LAPTM4B*-overexpressing vectors. Over-expression of *LAPTM4B* significantly augmented *HMOX1* induction by serum starvation (**Figure 7A**) as well as the nuclear accumulation of NRF2 (**Figure 7B**). It is noteworthy that while modulation of *LAPTM4B* expression did not affect levels of *HMOX1* in cells at basal conditions and cultured in FBS-containing medium (**Figures 6B and 7A**), *LAPTM4B* expression positively controlled nuclear levels of the transcription factor NRF2 (**Figures 6C and 7B**). It is reasonable to surmise that *HMOX1* induction by NRF2 is independent on the levels of the latter transcription factor but rather dependent on serum deprivation-induced stress. Our findings point to a novel intracellular mechanism, which involves the *LAPTM4B* field cancerization marker, for control of the NRF2 transcription factor during basal conditions and cellular stress (e.g. nutrient deprivation).

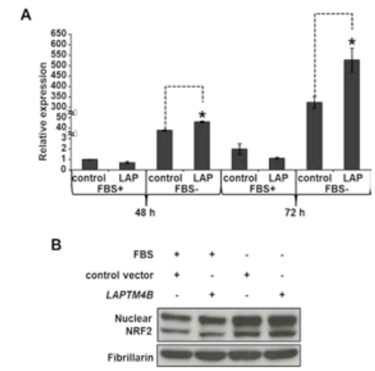

Figure 7. *LAPTM4B* over-expression augments *HMOX1* induction by serum starvation as well as NRF2 nuclear levels. A. QRTPCR analysis of *HMOX1* in cells transfected control and *LAPTM4B* overexpressing vectors in the presence and absence of FBS. **B**. Western blotting analysis of NRF2 levels in nuclear fractions. Membranes were incubated with antibody against the nucleolar marker fibrillarin to ensure equal loading. Error bars represent standard deviation. *, $P < 0.05$.

Studies exploring mechanisms of the oncogenic properties and function (**Figures 4-7**) of *LAPTM4B* field cancerization marker will be completed and then be prepared as a manuscript for publication which we anticipate submitting for peer-review within the next funding period.

C. Collection and transcriptome analysis of nasal and bronchial epithelia from patients with and without lung cancer (Sub-specific Aims 1A and 1C)

As detailed in our previous annual report (Year 2), epithelial brushings were being collected from the nasal compartment and from the airway (3 bronchial brushes). Tumor, normal lung tissue and airway samples were also collected from specimens resected following surgical lobectomy procedures. Lung tumors, normal lung tissue and airway samples from cases with lung cancer were obtained from all four participating institutions and samples from cases without lung cancer were collected and processed from BU (Partnering PI, Dr. Avrum Spira), Vanderbilt (Partnering PI, Dr. Pierre Massion) and UCLA (Initiating PI, Dr. Steve Dubinett). Total RNA from all samples in the different institutions were isolated similarly using the miRNeasy kit from Qiagen according to the manufacturer's instructions.

During the past year (Year 3), we started studying the molecular spatial map of field effects that transverse the normal-appearing bronchus adjacent to tumors up to the relatively distant nasal epithelium (**Figure 8A**). We surmised that this analysis would aid in identification of shared genomic changes between the field and lung cancer and that extend to compartments (e.g. nasal) in the field cancerization that can be readily accessible for biomarker analysis in screening and clinical settings. Samples (n=254) from patients with (n=28) and without (n=9) lung cancer that were collected from all partnering institutions (14 cancer cases from MD Anderson) were processed for global expression profiling at MD Anderson Cancer center using the Human Gene 2.0 ST platform (Affymetrix) and data analysis was performed in collaboration with BU (Partnering PI, Dr. Avrum Spira). We identified profiles differentially expressed between NSCLCs and benign nodules which were then analyzed by GSEA. The top 50 gene sets significantly (*P* <0.01) enriched in the NSCLC tumors were then analyzed and plotted across different spatial points in the field of injury/cancerization. Spatial analysis of molecular field profiles pointed to gene sets that decreased in tumor-associated enrichment with increasing distance of samples from tumors and those (highlighted in red) that remain enriched up to the nasal epithelium (**Figure 8B**). These data point to specific tumor-associated profiles that are enriched in the nasal epithelium and, thus, comprise readily accessible field cancerization markers for lung cancer detection. We are currently completing the microarray data analysis and subsequent validation studies which we expect to prepare as a manuscript and submit for publication within the next funding period.

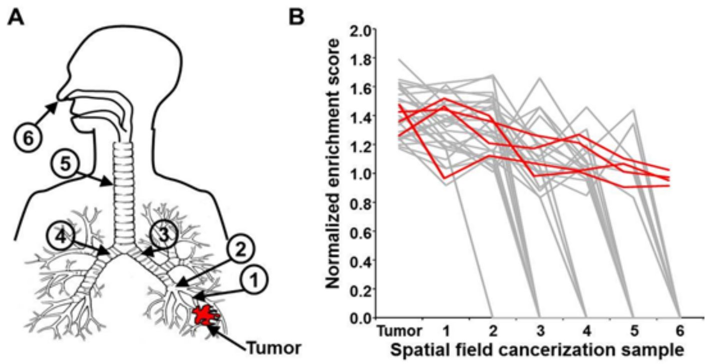

Figure 8. Spatial mapping of the field cancerization in NSCLC. Profiles in lesions (NSCLC tumors and benign nodules), airways adjacent to lesions (1 and 2), ipsilateral (3) and contralateral (4) main stem bronchi, tracheas (5) and nasal epithelia (6) were compared between NSCLC patients and those with benign disease (**A**). **B**. Profiles that were differentially expressed between airways 1 and 2 from patients with and without lung cancer were then analyzed by GSEA. The top 50 positively enriched (in cancer patient) pathways were then plotted for their enrichment across different spatial locations (1-6) in the field of cancerization. Plots indicate NSCLC-associated gene sets that decrease in positive enrichment in samples with increasing distance from tumors (grey) or remain highly enriched up the nasal epithelium (red).

Specific Aim 2: To increase our understanding of the role of tumor-initiating stem/progenitor cells in the pathogenesis of lung cancer in the "field cancerization" that develops in current and former smokers.

<u>Summary of Research Findings:</u>

Dr. Wistuba's laboratory is not part of this Aim.

Specific Aim 3: Test airway-based mRNA and microRNA biomarkers of diagnosing lung cancer in current and former smokers at high risk for lung cancer in minimally invasive sites.

<u>Summary of Research Findings:</u>

Due to the use of both next generation RNA sequencing and comprehensive microarray profiling and due to this ongoing study's unique design we anticipate that expression profiles in the NSCLC molecular field of injury will harbor molecules, both novel and established, that may exhibit potential for use as airway biomarkers that can be developed and tested for lung cancer detection using minimally invasive sites in Specific Aim 3 of this award. As mentioned in Specific Aim 1 above, we have identified profiles in the field of injury/cancerization that are also enriched in the nasal compartment of patients with lung cancer relative to patients with benign disease. While microarray (MD Anderson and BU) and RNA sequencing (RNA-seq) (BU) data analyses are being completed, **all four sites/institutions are continuing to collect** nasal and airway samples from patients with lung cancer and BU, Vanderbilt and UCLA are continuing to collect nasal and airway samples from patients without lung cancer or benign disease. **These new cases** will serve as sets to develop and validate classifiers, that are based on profiles from Aim 1 as mentioned above, that can be analyzed readily in the clinic (e.g. by QRTPCR) in minimally invasive sites (e.g. nasal compartment) in smokers with indeterminate nodules. In addition, to the fourteen lung cancer cases that have already been profiled in Specific Aim 1, as mentioned above, **we have collected at MD Anderson Cancer Center additional 43 lung cancer cases comprised of large airways, airways adjacent to the nearby lung tumor and nasal epithelia**. These additional cases will be utilized for development of the classifier in **Year 4** of the grant period.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the transcriptomic architecture of the adjacent airway field cancerization in early-stage non-small cell lung cancer. These analyses demonstrated that the adjacent airway field of cancerization is comprised of markers that can identify lung cancer among smokers as well as gradient and localized site-dependent expression patterns that recapitulate NSCLC profiles. These findings have been submitted recently for publication and are under revision.
- Demonstrated for the first time that the field cancerization putative oncogene, *LAPTM4B*, is a positive mediator of the lung cancer cell malignant phenotype evidenced by its promotion of anchorage-independent colony formation in soft agar.
- Studied the mRNA expression of *LAPTM4B* in a large series of NSCLC histological tissue specimens for the first time by *in situ* hybridization. This analysis revealed that *LAPTM4B* expression is significantly positively associated with smoking and worse overall survival.
- Demonstrated that the field cancerization marker *LAPTM4B* protects lung cancer cells from serum deprivation-induced growth inhibition and promotes the autophagy response following serum deprivation.
- Revealed that *LAPTM4B* is a novel positive regulator of NRF2 transcription factor in lung cancer cells.
- In collaboration with the Partnering PIs and Initiating PI of this grant, performed microarray profiling at MD Anderson of 254 field cancerization samples from 28 cases with lung cancer and 9 cases with benign disease to begin to characterize the molecular spatial map of field effects that transverse the bronchus adjacent to tumors up to the nasal epithelium. This novel analysis demonstrated that the molecular map of the field of injury/cancerization, in patients with lung cancer, is comprised of pathways and gene sets, whose enrichment in the field decreases with larger distance from the tumor as well as those that persist up to the nasal epithelium.

REPORTABLE OUTCOMES

Abstracts:

- Maki Y, Fujimoto J, Yoo SY, Gower A, Shen L, Garcia MM, Kabbout M, Chow CW, Hong WK, Kalhor N, Wang J, Moran C, Spira A, Coombes KR, Wistuba II, Kadara H. Transcriptomic architecture of the airway field cancerization in early-stage non-small cell lung cancer. 104th Annual American Association for Cancer Research (AACR) meeting, April 6 - April 10 2013, Washington, D.C. Abstract # 2367.

Manuscripts:

- Kadara H, Fujimoto J, Yoo SY, Maki Y, Gower AC, Kabbout M, Garcia MM, Chow CW, Chu Z, Mendoza G, Shen L, Kalhor N, Hong WK, Moran C, Wang J, Spira A, Coombes KR, Wistuba II. Transcriptomic architecture of the adjacent airway field cancerization in non-small cell lung cancer. *Journal of the National Cancer Institute*. Submitted and Under Revision.

CONCLUSIONS

During our third year of research, we characterized the transcriptomic architecture of the adjacent airway field cancerization in early-stage NSCLC. Our studies demonstrated that the adjacent airway field of cancerization is comprised of markers that can identify lung cancer among smokers as well as gradient and localized site-dependent expression patterns that recapitulate NSCLC profiles. Our findings on the adjacent field of cancerization provide additional insights into the biology of NSCLC and the development of molecular tools for the detection of the malignancy. Furthermore, we studied in detail the functional roles and properties of *LAPTM4B*, a putative oncogene that we identified as a field cancerization marker, in lung cancer pathogenesis. We found that *LAPTM4B* promotes growth of lung cancer cells in soft agar, is associated with smoking and worse overall survival in NSCLC, protects cancer cells from serum deprivation-induced growth inhibition and positively controls the autophagic response and the NRF2 transcription factor during cellular stress. Furthermore, we began to characterize the molecular spatial map of field effects that transverse the bronchus adjacent to tumors up to the nasal epithelium. This novel analysis identified field of injury/cancerization pathways and gene sets, in patients with lung cancer, which decrease in enrichment with larger distance from the tumor as well as those that persist up to the nasal epithelium. These data point to specific tumor-associated profiles that are enriched in the nasal epithelium and, thus, comprise readily accessible markers for lung cancer detection that will be further refined and validated in the next year of the funding period.
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REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.
2. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008;359:1367-80.
3. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer. 1953;6:963-8.
4. Steiling K, Ryan J, Brody JS, Spira A. The field of tissue injury in the lung and airway. Cancer Prev Res (Phila Pa). 2008;1:396-403.
5. Wistuba, II, Gazdar AF. Lung cancer preneoplasia. Annu Rev Pathol. 2006;1:331-48.

6. Powell CA, Klares S, O'Connor G, Brody JS. Loss of heterozygosity in epithelial cells obtained by bronchial brushing: clinical utility in lung cancer. Clin Cancer Res. 1999;5:2025-34.
7. Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, Palma J, Brody JS. Effects of cigarette smoke on the human airway epithelial cell transcriptome. Proc Natl Acad Sci U S A. 2004;101:10143-8.
8. Spira A, Beane JE, Shah V, Steiling K, Liu G, Schembri F, Gilman S, Dumas YM, Calner P, Sebastiani P, Sridhar S, Beamis J, Lamb C, Anderson T, Gerry N, Keane J, Lenburg ME, Brody JS. Airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer. Nature medicine. 2007;13:361-6.
9. Sridhar S, Schembri F, Zeskind J, Shah V, Gustafson AM, Steiling K, Liu G, Dumas YM, Zhang X, Brody JS, Lenburg ME, Spira A. Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. BMC Genomics. 2008;9:259.
10. Li Y, Iglehart JD, Richardson AL, Wang ZC. The amplified cancer gene LAPTM4B promotes tumor growth and tolerance to stress through the induction of autophagy. Autophagy. 2012;8:273-4.
11. Li Y, Zhang Q, Tian R, Wang Q, Zhao JJ, Iglehart JD, Wang ZC, Richardson AL. Lysosomal transmembrane protein LAPTM4B promotes autophagy and tolerance to metabolic stress in cancer cells. Cancer research. 2011;71:7481-9.
12. Li Y, Zou L, Li Q, Haibe-Kains B, Tian R, Li Y, Desmedt C, Sotiriou C, Szallasi Z, Iglehart JD, Richardson AL, Wang ZC. Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer. Nature medicine. 2010;16:214-8.
13. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003;4:249-64.
14. Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. Nature reviews Cancer. 2012;12:564-71.
15. Kweon MH, Adhami VM, Lee JS, Mukhtar H. Constitutive overexpression of Nrf2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate. The Journal of biological chemistry. 2006;281:33761-72.

APPENDICES

Transcriptomic architecture of the airway field cancerization in early-stage non-small cell lung cancer

Yuho Maki, Junya Fujimoto, Suk-Young Yoo, Adam Gower, Mohamed Kabbout, Li Shen, Melinda M. Garcia, Chi-Wan Chow, Waun K, Hong, Neda Kalhor, Jing Wang, Cesar Moran, Avrum Spira, Kevin R. Coombes, Ignacio I. Wistuba, Humam Kadara

Earlier work has identified in lung cancer a field cancerization (FC) phenomenon in which tumors and adjacent normal appearing tissues share specific molecular abnormalities (e.g., loss of heterozygosity) that may be highly pertinent to cancer pathogenesis. We sought to characterize the global molecular airway FC adjacent to early-stage non-small cell lung cancer (NSCLC) in an attempt to unravel profiles that may help to explain the development of the disease.

We performed whole-transcript expression profiling of a set of resected early-stage NSCLC specimens (n=20 patients) with matched histologically normal airways of varying distance from the tumor and paired uninvolved normal lung tissue (n=194 samples). Using linear mixed-effects models, we derived FC profiles signifying genes concordantly differentially expressed between tumors and airways compared to normal lung tissues. Gene set enrichment analysis demonstrated that a subset of the genes (n=299) was significantly and congruently modulated between large airways of smokers with and without lung cancer. We then questioned whether the airway FC exhibits site from tumor-dependent expression patterns. Ordinal regression analysis identified airway profiles (n=422 genes) that were significantly progressively expressed by distance from tumors and topologically organized into canonical cancer-associated pathways, such as eukaryotic initiation factor, p70S6K kinase, polo-like kinase and mammalian target of rapamycin signaling (all $p<0.001$). In addition, the site-dependent airway profiles recapitulated NSCLC expression patterns and were concordantly modulated between tumors and uninvolved normal lung tissues pinpointing their probable roles in lung cancer pathogenesis. Quantitative real-time PCR (QRTPCR) analysis confirmed the differential expression of FC markers selected by both pathways analysis and statistical criteria. Notably, lysosome associated protein transmembrane 4 beta (*LAPTM4B*), a putative oncogene with no known role in lung carcinogenesis, was among the top 5 site-dependent FC markers and was significantly elevated in NSCLC and immortalized bronchial epithelial cell lines compared to normal cells. Furthermore, transient or stable knockdown of *LAPTM4B* by RNA interference decreased NSCLC cell growth as well as anchorage-dependent and -independent colony formation.

In conclusion, our efforts in understanding the adjacent molecular FC in NSCLC unraveled airway profiles that 1) are, in part, relevant to lung cancer detection; 2) are modulated by distance from corresponding tumors; 3) recapitulate NSCLC expression patterns and 4) harbor markers engaged in mediating the lung malignant phenotype. Profiling the adjacent airway cancerization, in conjunction with tumors, may provide additional insights into the molecular pathology of NSCLC. Funded in part by Department of Defense award W81XWH-10-1-1007.

THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History

Ignacio I Wistuba, M.D.
Professor and Chair
Anderson Clinical Faculty Chair for
Cancer Treatment and Research
Department of Translational Molecular
Pathology
2130 W Holcombe Blvd
LSP9.4029, Unit 2951
Houston, TX 77030
T 713-563-9184, F 713-384-6082
iiwistuba@mdanderson.org

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Re: Grant – W81XWH-10-1-1007
"Molecular Profiles of Lung Cancer Pathogenesis and Detection in U.S. Veterans"

This letter is to inform the sponsor of personnel changes for the fourth year of the aforementioned project. Dr. Edward Kim and Dr. Waun Hong were previously on the project at 2% and 1% effort, however for this current year, they will no longer be on the project. Additionally, Dr. Humam Kadara's effort will be adjusted from 50% to 25% effective September 1, 2013.

The change in level of effort is feasible since the project is moving in a satisfactory direction and the aims of the project are being met.

No rebudgeting will be required as a result of this change in effort.

Thank you for your attention and consideration in this matter.

Ignacio Wistuba, M.D.
Professor & Chair
Department of Translational Molecular Pathology
UT MD Anderson Cancer Center